ZOOLOGICAL SCIENCE 11: 261–267 (1994)

Control of Growth and Differentiation of Chondrogenic Fibroblasts in Soft-Agar Culture: Role of Basic Fibroblast Growth Factor and Transforming Growth Factor-β

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ABSTRACT—The sclera of the chick embryo consists of a layer of cartilage cells (scleral chondrocytes) adjacent to a layer of perichondrium (chondrogenic fibroblasts), which can be separated to produce pure populations of each cell type. In soft-agar culture at low concentration of fetal bovine serum (FBS), basic fibroblast growth factor (bFGF) induced clonal growth of many undifferentiated fibroblast-type (F-type) colonies from chondrogenic fibroblasts. Under the same conditions, bFGF induced many differentiated cartilage-type (C-type) colonies from scleral chondrocytes. On the other hand, a high concentration (10%) of FBS induced many C-type colonies from the chondrogenic fibroblasts. These results indicate that bFGF induces the fibroblasts to proliferate without progression of differentiation, while FBS contains an activity which promotes cartilage differentiation of the fibroblasts. The proliferating fibroblasts retained their differentiative capacity for at least 20 days in culture. The bFGF-dependent proliferation of the chondrogenic fibroblasts was inhibited by low concentration of transforming growth factor-β (TGF-β). In contrast, with differentiated chondrocytes, TGF-β did not inhibit the bFGF-dependent proliferation, but promoted it synergistically. Conditioned medium harvested from protein-free monolayer cultures of chondrogenic fibroblasts contained a TGF-β-like molecule. These regulators may play roles in the growth and differentiation of chondrogenic cells *in vivo*.

INTRODUCTION

Locally accumulating growth factors, such as bFGF and TGF-β, have been demonstrated to play morphogenetic roles in chondrogenesis [2, 8, 9, 12]. However, little information is available on the effects of these factors on undifferentiated and differentiated cells of the same cell lineage. Moreover, there has been little work to isolate candidate autocrine regulators directly from the cells involved in differentiative pathways.

To approach this problem, it is necessary to isolate pure cell populations representing different differentiative states, especially the undifferentiated cells. In addition, it is necessary to know the basal *in vitro* conditions for the cells to proliferate with retention of differentiative capacity and to differentiate only when certain signals are applied. Ideally, these cells should also be able to serve as a source of regulatory factors from their conditioned medium.

Recently, we have established an experimental system from the chick sclera [5, 19, 20]. The skeleton of the chick eyeball is composed of the scleral cartilage layer and its perichondrium, the scleral fibroblast layer, both of which are derived from neural crest cells [17]. These two layers are easily separable into two sheets without mutual contamination. The scleral chondrocytes manifest a cartilage phenotype in monolayer culture [20], while the scleral fibroblasts (chondrogenic fibroblasts) are motile, flattened fibroblasts which show no detectable expression of a cartilage phenotype, but proliferate in protein-free medium by producing multiple growth-regulating factors [5, 11, 19]. However, when scleral fibroblasts are cultured in soft agar containing 10% FBS, the cells proliferate and differentiate into rounded chondrocytes [20]. This suggests that the scleral fibroblasts represent a population of undifferentiated chondrogenic fibroblasts, which, although normally fibroblasts, have the capacity to differentiate into chondrocytes when suitable conditions are provided.

In this report, we describe the differential roles of low concentrations of bFGF, TGF-β and serum on chondrogenic fibroblasts or their differentiated progeny, scleral chondrocytes, in serum-deprived soft-agar culture. In addition, we tried to prove the presence of TGF-β-like molecule in conditioned medium harvested from protein-free primary cultures of the same cell type.

MATERIALS AND METHODS

1. Isolation of scleral fibroblasts and scleral chondrocytes: Dissection from embryos and dissociation of cells

Procedures have been published previously [11, 19, 20]. In brief, scleras were isolated from the eyeballs of 12-day chick embryos, and scleral fibroblast layers and scleral cartilage layers were separated under a microsurgery microscope. The isolated tissues were incubated with 0.2% collagenase (*Clostridium histolyticum*, activity 150–300 units/mg, Wako Ltd., Japan) at 37°C, for 40 or 60 min. The softened tissues were pipetted gently two times in culture medium, and the resulting cell suspensions were filtered through 2 sheets of sterile gauze and confirmed to be 99% single cells.

Accepted March 24, 1994
Received March 9, 1994

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2. Serum-deprived soft-agar culture and supplementation of test agents

One volume of a 1% aqueous solution of agar (Agar Noble, Difco Laboratories, U.S.A.) was mixed with one volume of 2-fold concentrated Ham's F-12 medium (Nissui Pharmaceutical Co., Japan) at 40–42°C to produce a 0.5%-agar solution in F-12 medium (hard-agar solution). Aliquots of 1 ml of the solution were poured into 35-mm plastic culture dishes (Falcon 3001, Becton Dickinson, U.S.A.) and hardened at room temperature to make a hard-agar layer.

Next, one volume of F-12 medium containing dissociated cells was mixed with two volumes of hard-agar solution at 40–42°C to produce a cell suspension in 0.33%-agar (soft-agar) solution. For each culture, 1 ml of the soft-agar solution containing 2×10^3 cells was quickly poured onto the hard-agar layer. Both of the agar layers were protein-free. After the agar layers hardened, 1 ml of F-12 medium containing the test agents with different concentrations of FBS (GIBCO Laboratories, U.S.A.) was poured onto the soft-agar layer. The cultures were incubated at 38°C in 5% CO_2-moist air, without any supplementation of test agents or serum. Colony-forming effieciency (number of resultant colonies per incubated cell number $\times100$) was calculated after 20 days of culture. The number of F-type colonies [20] was counted; only those F-type colonies larger than 50 μm in diameter were scored. In C-type colonies [20], colonies containing more than 6 cells were counted.

3. Assays for cell differentiation in soft-agar culture: Alcian blue staining and immunohistochemical staining

The culture dishes were dried at 55°C for 60 min and fixed with 10% formalin for 3 hr. Cells were stained with 0.1% Alcian blue dissolved in 0.1 N HCl for 3 hr at room temperature.

Anti-PG-H rabbit serum (provided by Dr. Koji Kimata, Aichi Medical University) was used for detecting cartilage-specific proteoglycan, PG-H [15]. Indirect immunohistochemical staining with biotinylated secondary antibody and β-galactosidase-bound streptavidin was performed as described previously [20].

4. Preparation of conditioned medium from protein-free monolayer culture of the scleral fibroblasts and ELISA of the conditioned medium material for anti-TGF-β immunoreactivity

Methods for preparing protein-free monolayer culture and harvesting conditioned medium were given elsewhere [19]. The harvested conditioned medium was concentrated from 50 ml to 4 ml by centrifugal concentrator (VC-360, Taitec Co., Japan). The concentrate and precipitate were mixed, transferred into membrane tubing (Dialysis Membrane, Size 20; cut off 10 kDa, Waco Ltd.) and dialysed against distilled water for 5 days. After centrifugation at 12,000 rpm for 20 min, the supernatant was further 4-fold concentrated and used as the test sample.

Aliquots of 50 μl of test samples or control TGF-β_1 (serial dilution from 100 ng/ml) were diluted 2-fold with 0.1 M carbonate-bicarbonate buffer (pH 9.5) and immobilized onto a 96-well microplate for ELISA (Japan Intermed Co., Japan) by incubation at 4°C overnight. The plate was washed 3 times with 0.05% Tween-20 (Bio-Rad Lab.) in PBS (TPBS), blocked with 200 μl of 1% gelatin-containing PBS at 37°C for 1 hr, and wahed with TPBS 3 times. A 50-μl aliquot of 150-fold diluted rabbit anti-human TGF-β_1 antibody (IgG; King Brewing Co., Japan) was added to each well. After 30 min, the plate was washed with TPBS 5 times, and peroxidase-labelled goat anti-rabbit IgG (H+L) (Kirkegaard and Perry Lab. Co.) was added to each well. After 1 hr, the plate was again washed with TPBS 5 times and 100 μl of peroxidase substrate solution was added to each well. After 30 min, the reaction was stopped by adding 50 μl of 2 M H_2SO_4. Absorbance at 490 nm was measured with a microplate reader (Bio-Tek, EL-309). As a control for specific antibody, rabbit IgG (Zymed Lab.) was used.

5. Growth factors

Basic FGF (bFGF, purified from bovine brain) was purchased from R & D systems, U.S.A. TGF-β (recombinant human TGF-β_1) was from king Brewing Co., Japan. Insulin (purified from bovine pancreas) was from Sigma Chemical Co., U.S.A. Platelet-derived growth factor (purified from human leucocytes) was from Collaborative Research Inc., U.S.A.

RESULTS

1. Effects of serum deprivation of chondrogenic fibroblasts in soft-agar culture

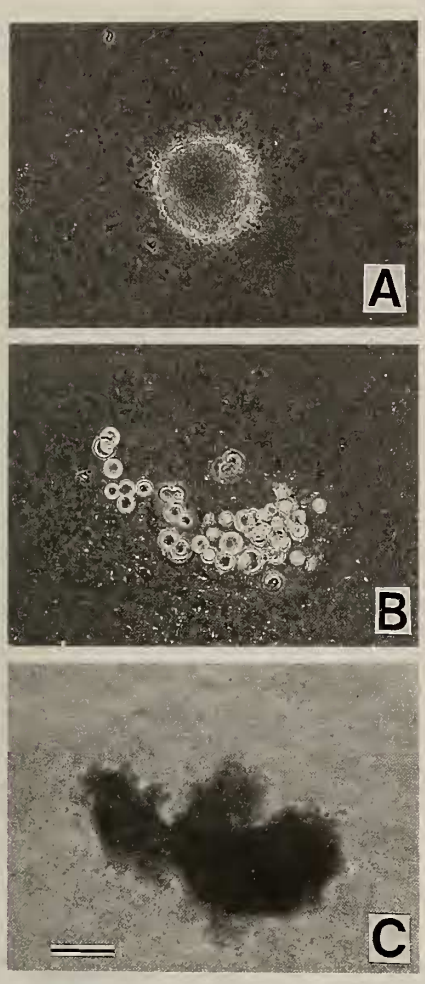
Chondrogenic fibroblasts in soft-agar culture in 10% FBS gives rise to two types of clonal colonies [20]. The F-type colony is round and consists of mutually adhering flattened fibroblasts (Fig. 1A), and the C-type colony consists of scattered large chondrocytes (Fig. 1B) with a halo of cartilage matrix, which is positive to staining with Alcian blue (Fig. 1C) or anti-PG-H antibody [20]. The colony-forming efficiency decreases by lowering serum concentration (Fig. 1D). Because no colonies were observed in serum-free medium, even in the presence of various growth factors, the analysis of the effects of different factors were performed in medium containing 2% FBS.

2. Basic FGF induces colony formation without promotion of differentiation, and FBS promotes chondrogenic differentiation

As shown in Table 1, 10 ng/ml bFGF induces many large F-type colonies in chondrogenic fibroblasts, which suggests that, relative to TGF-β, PDGF and insulin, bFGF does not promote cartilage differentiation by the proliferating fibroblasts. The number of F-type colonies increases and that of C-type colonies decreases as a function of bFGF concentration (Table 1).

FBS at 10% is effective at promoting C-type colonies, which suggests that FBS contains a strong activity for induction of cartilage differentiation from the fibroblasts (Table 1). The bFGF-induced large F-type colonies can differentiate into C-type colonies (C-type conversion) [20], if the culture medium changed on day 20 to fresh medium containing 2% FBS without bFGF and the cultures are maintained for further 14 days (Fig. 2). This suggests that cells proliferating in the presence of bFGF retain their differentiative capacity for at least 20 days.

With differentiated chondrocytes from the scleral cartilage layer, bFGF also induces rapid proliferation to form many colonies (data not shown). In this case, cell size remains smaller (mean diameter 10 μm) than in the absence of bFGF (mean diameter 30 μm); this suggests that there is no hypertrophic differentiation, which takes place in the



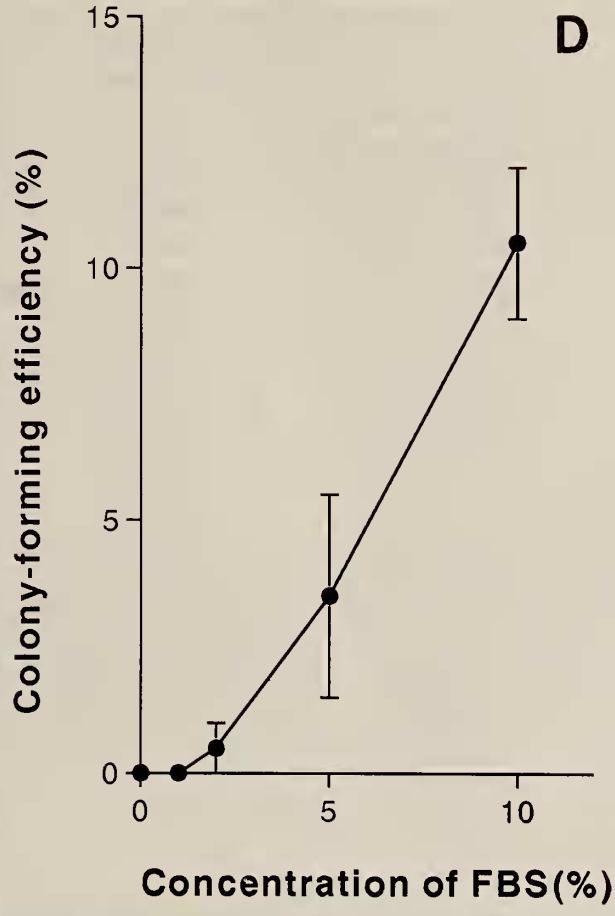


FIG. 1. Colony formation of chondrogenic fibroblasts (scleral fibroblasts) in soft-agar culture and the effects of serum concentration. A: F-type colony. B: C-type colony. Both photographs are the same magnification, suggesting large cell size in C-type colony. A and B were observed on the day 20 of culture by phase-contrast microscope. C: The same colony as B, stained with Alcian blue, showing a halo of cartilage matrix surrounding individual chondrocytes, as described previously [20]. Bar, 100 μm. D: Effects of serum (FBS) concentration on colony formation. Each point represents the average of results from three dishes with standard deviation.

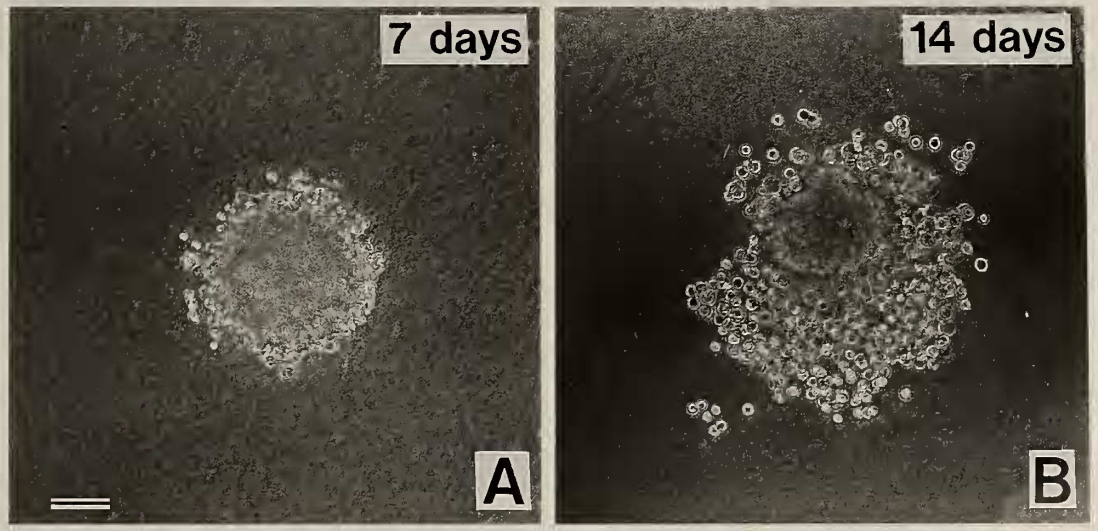


FIG. 2. Chondrocyte differentiation (C-type conversion) from bFGF-dependent large F-type colony. After culturing for 20 days with 10 ng/ml bFGF, the medium was changed to fresh medium containing 2% FBS and no bFGF. The culture was further incubated for 14 days. The same colony was photographed at the 7th day (A) and 14th day (B) after medium change. Rounded cells, which migrate out from the periphery of the F-type colony, are differentiating chondrocytes (C-type conversion, as described previously) [20]. About 5% of the bFGF-dependent F-type colonies manifested the C-type conversion. Bar, 100 μm.

TABLE 1. Effects of various growth-promoting substances on colony formation by chondrogenic fibroblasts in soft-agar culture

	Number of colonies obtained[a]	C-type colony	F-type colony	LF-type colony[b]
(Experiment A)[c]				
None (2% FBS only)	80 (4)	13	67	0
bFGF (10 ng/ml)	780 (39)	0	780	585
TGF-β (1 ng/ml)	140 (7)	10	130	67
PDGF (3 U/ml)	100 (5)	3	97	30
Insulin (10 μg/ml)	140 (7)	36	104	73
10% FBS	420 (21)	311	109	0
10% FBS with bFGF[d]	1960 (98)	510	1450	1058
(Experiment B)				
None (2% FBS only)	60 (3)	30	30	0
bFGF (0.01 ng/ml)	200 (10)	30	170	68
(0.1 ng/ml)	220 (11)	33	187	103
(1 ng/ml)	180 (9)	18	162	97
(10 ng/ml)	280 (14)	0	280	126

a: Dissociated cells were inoculated at 2000 cells per dish. Mean values obtained from three dishes are shown. Parenthesis means colony-forming efficiency.
b: LF-type (large F-type) colony means F-type colonies with diameters more than 81 μm.
c: Experiment A and Experiment B were different sets of cell culture. Growth factors were supplemented together with 2% FBS unless otherwise indicated. The variation in the results between these two experiments is as expected for different sets of cultures.
d: bFGF was added at 10 ng/ml.

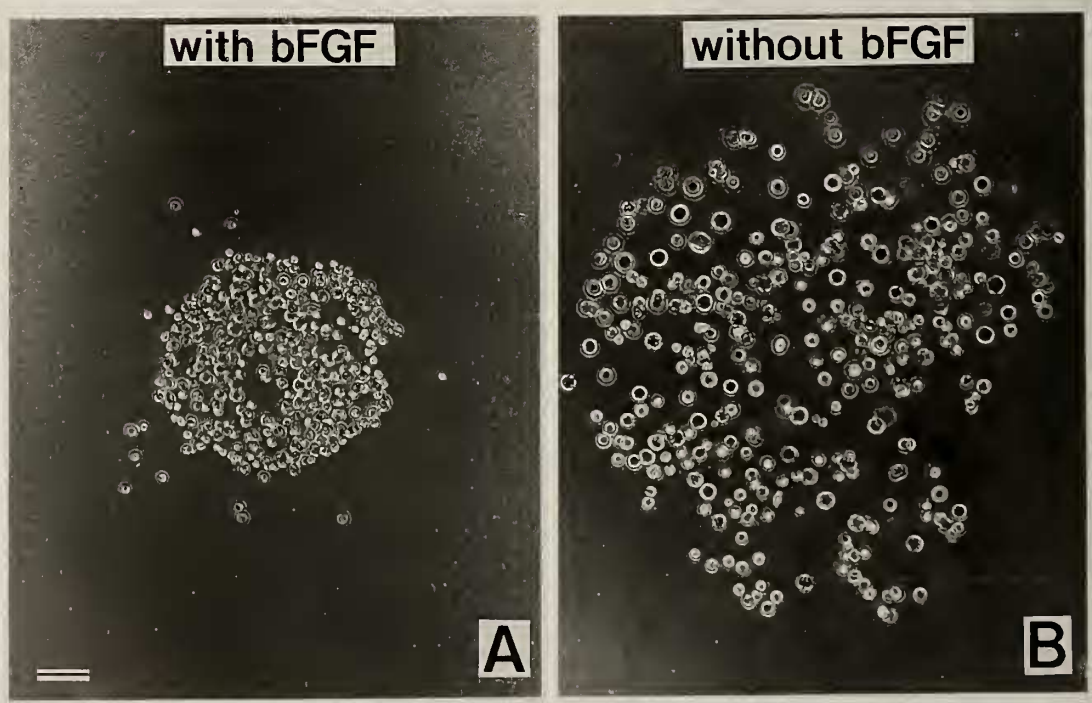


FIG. 3. Two different types of C-type colonies derived from differentiated chondrocytes. A: In the presence of 1 ng/ml bFGF. B: In the absence of bFGF. Both photographs are the same magnification. Both cultures contain 10% FBS. Photographed on day 20 of culture. Bar, 100 μm.

absence of bFGF (Fig. 3) [7].

3. TGF-β modulates the bFGF-dependent proliferation depending on the state of differentiation

Supplementation with bFGF alone raises colony-forming efficiency of both chondrogenic fibroblasts (●; Fig. 4A) and differentiated chondrocytes (●; Fig. 4C). However, supplementation with TGF-β alone does not increase it for either cell type (▲; Fig. 4B, D).

When TGF-β is supplemented with 1 ng/ml bFGF, TGF-β inhibits bFGF-dependent colony formation by chondrogenic fibroblasts in a dose-dependent manner at a range of 0.001–1 ng/ml (△; Fig. 4B). In contrast, in the presence of bFGF, TGF-β increases cloning efficiency by differentiated chondrocyte at the same range of concentrations (△; Fig. 4D). TGF-β at these concentrations does not affect cellular differentiation in soft-agar culture (data not shown).

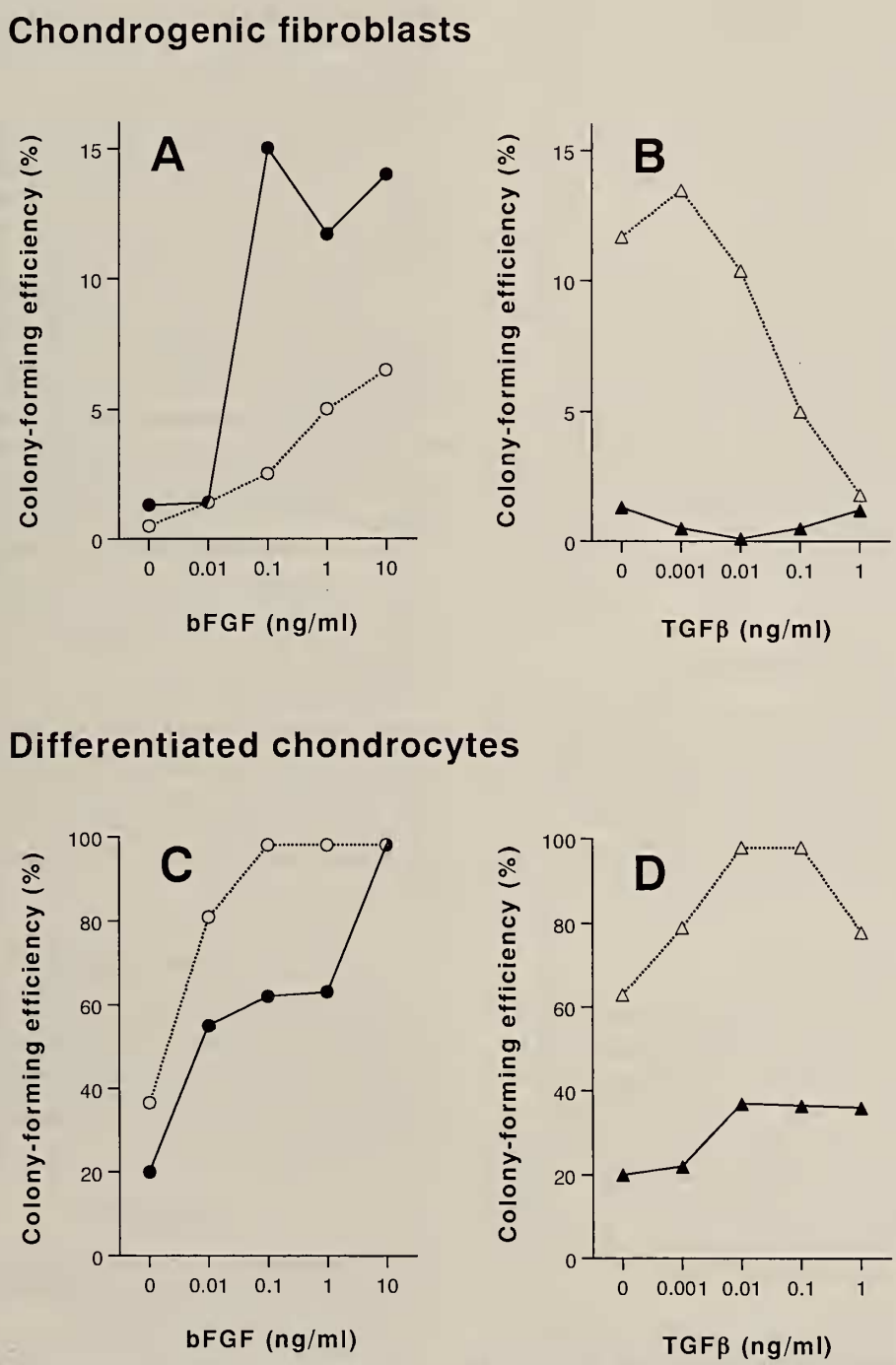


FIG. 4. Effects of bFGF and TGF-β on colony formation. A and B: In the case of chondrogenic fibroblasts. C and D: In the case of differentiated chondrocytes. Solid circles (●): bFGF alone. Open circles (○): bFGF with TGF-β (0.1 ng/ml). Solid triangles (▲): TGF-β alone. Open triangles (△): TGF-β with bFGF (1 ng/ml). Mean values obtained from three dishes are plotted.

4. Presence of anti-TGF-β immunoreactivity as autocrine/paracrine regulators

Assay of concentrated conditioned medium by ELISA with anti-TGF-β_1 antibody indicates that conditioned medium contains a TGF-β-like molecule (Fig. 5). The amount in conditioned medium seems to be higher at the early days of culture.

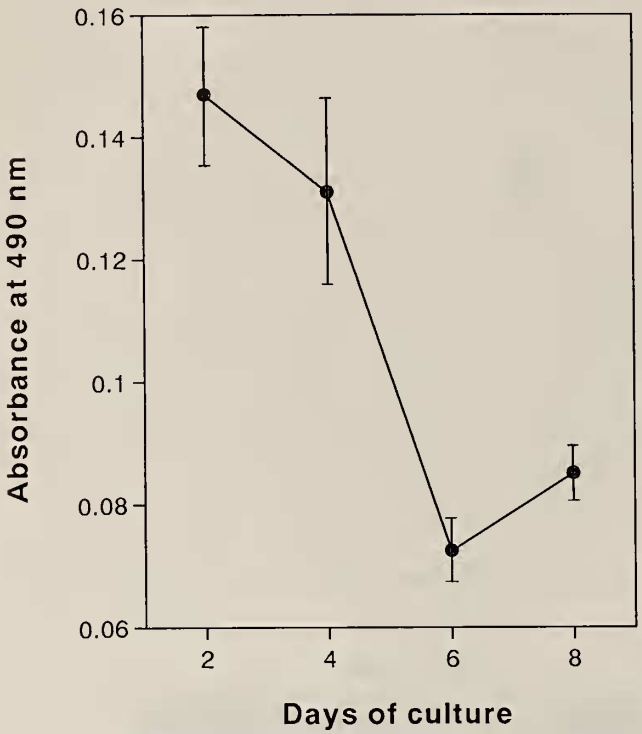


Fig. 5. Detection of TGF-β-like molecule in conditioned medium of chondrogenic fibroblasts in monolayer culture by ELISA with anti-TGF-β_1 antibody. Concentrated conditioned medium was immobilized onto wells (see Materials and Methods). Absorbance of the negative control (non-immune rabbit IgG) was 0.023, and that of the positive control (25 ng/ml TGF-β_1) was 0.127. Data given are the average values from 3 replicate wells with standard deviation.

DISCUSSION

With chondrogenic fibroblasts (scleral fibroblasts), bFGF [6] induced rapid proliferation to produce many large F-type colonies (Table 1). Since many of the chondrogenic fibroblasts possess the capacity to proliferate and differentiate into C-type colonies when 10% FBS is present [20] (Table 1), bFGF was considered to have a capacity to induce these cells to proliferate without promotion of differentiation. The differentiative potentiality of the proliferating cells was maintained even up to 20 days after initial treatment with 10 ng/ml bFGF (Fig. 2).

With differentiated chondrocytes (scleral chondrocytes), bFGF also induced rapid proliferation with suppression of hypertrophic differentiation (Fig. 3), similar to the case for rabbit chondrocytes [7, 8]. The suppression of differentiation by bFGF does not imply inhibition of phenotypic expression. In fact, Kato [8] pointed out that bFGF stabilized phenotypic expression and prevented dedifferentiation of proliferating chondrocytes, and, as a result, promoted proteoglycan synthesis.

Taken together, the function of bFGF is to induce proliferation of both undifferentiated and differentiated chondrogenic cells without promotion of differentiation, i.e., to expand a cell population *as it exists*. We emphasize that an initial treatment with bFGF is sufficient for a persistent effect for at least 20 days. This may suggest the existence of a mechanism to hold a cellular differentiative state steady during persistent proliferation, similar to the auto-induction in steroid-hormonal regulation [18].

As to the differentiation-promoting activity, which at least FBS possessed, bone morphogenetic proteins (BMPs) [14] have been reported to be differentiation factors in limb bud mesoderm of the chick [3, 4]. In fact, recombinant BMP-4 has been found to induce C-type conversion (Watanabe, Hayashibe and Takaoka, unpublished data). It would be of interest to determine, whether FBS contains a member of the BMP family or stimulates autocrine production of one.

As shown in Figure 4, TGF-β [10, 13] displays an inverse effect on the bFGF-dependent proliferation of chondrogenic fibroblasts and differentiated chondrocytes, which are located adjacent to each other *in vivo*. Since our three-dimensional cultures should reflect the *in vivo* function of chondrogenic cells [1, 16], it may be that increased endogenous TGF-β-like molecule gives rise to overall growth of cartilage tissue, together with growth suppression of the adjacent perichondrium, in the presence of endogenous bFGF-like growth-promoting activity [19].

It was found that the chondrogenic fibroblasts secreted TGF-β-like molecule into their conditioned medium (Fig. 5). High activity at the early days in culture might be explained as an induction by primary monolayer cultivation. It is plausible that these factors play regulatory roles in the differential growth and differentiation of chondrogenic cells in *in vivo* scleral chondrogenesis.

ACKNOWLEDGMENTS

We thank Prof. Minoru Amano for his continuous encouragement and Dr. Akira Kawahara for his kind advice. Thanks are also due to Dr. David A. Carrino for reviewing manuscript. This work was supported in part by Grants-in-Aid for Scientific Research (No. 03833023 and No. 03304009) to K. W. from the Ministry of Education, Science and Culture of Japan.

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